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(71)(72) Applicants and Inventors: BAFOR, Maureen [NG/NG]; P.O. Box 4196, Benin City (NG). BANAS, Antoni [PL/PL]; ul. Wiolinowa 14, PL-08-110 Siedlce (PL). DAHLQVIST, Anders [SE/SE]; Hemmansvägen 2, S-244 66 Furulund (SE). GUMMESON, Per-Olov [SE/SE]; Björnbärsstigen 5, S-227 38 Lund (SE). LEE, Michael [NZ/SE]; Storgatan 11, S-231 97 Klagstorp (SE). LENMAN, Marit [SE/SE]; Revingegatan 13 A, S-223 59 Lund (SE). SJÖDAHL, Staffan [SE/SE]; Valthornsvägen 22, S-756 50 Uppsala (SE). STYMNE, Sten [SE/SE]; Torrlösa 1380, S-268 90 Svalöv (SE).

(74) Agents: ALDENBÄCK, Ulla et al.; Dr. Ludwig Brann Patentbyrå AB, P.O. Box 1344, S-751 43 Uppsala (SE).

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(57) Abstract

The present invention relates to a novel plant enzyme called delta (12) fatty acid acetylenase. This enzyme is responsible for the conversion of fatty acids to acetylenic acids and the invention relates to production of such acids. The invention also relates to use of cDNA encoding acetylenase, preferably *Crepis alpina* delta (12) acetylenase, for transforming organisms such as oil accumulating organisms selected from the group consisting of oil crops, oleogeneous yeasts and moulds. Furthermore, the invention relates to organisms such as oil accumulating organisms transformed with acetylenase cDNA, and to oils and other acetylenic compounds from said organisms.

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NOVEL PLANT ENZYME AND USE THEREOF

Technical field

The present invention relates to a novel plant enzyme. More specifically, the present invention relates to a method for producing acetylenic compounds in particular acetylenic fatty acids, to a cDNA encoding a plant fatty acid acetylenase, to the use of the said cDNA for transforming oil accumulating organisms for the purpose of producing acetylenic fatty acids, and to such oil accumulating organisms per se as well as oils therefrom.

Background of the invention

There is considerable interest, world-wide, in producing chemical feedstocks such as fatty acids for industrial use from renewable plant resources rather than from non-renewable petrochemicals. This concept has broad appeal for both manufacturers and consumers on the basis of resource conservation and in addition provides significant opportunities to develop new industrial crops for agriculture.

There is an enormous diversity of unusual fatty acids in oils from wild plant species which have been well characterized (see e.g. Badami & Patil, 1981). Many of these acids are of potential industrial use. This has lead to an interest in domesticating relevant plant species to enable the agricultural production of particular fatty acids. However the development of genetic engineering combined with a greater understanding of the biosynthesis of unusual fatty acids make it now possible to transfer genes coding for key enzymes, involved in the synthesis of a particular fatty acid from a wild species, to a choosen domesticated oilseed crop. In this way specific fatty acids can be produced in high purity and quantities at moderate costs.

One class of fatty acids of particular interest are the acetylenic fatty acids; consisting of an acyl chain having two

adjacent carbon atoms linked by an acetylenic or triple bond. Because of their high reactivities they may be ideally suited for the production of coatings, plastics and lubricants. By transferring the genes responsible for the production of a specific acetylenic acid from a wild species to commercial oilseeds, or any other oil accumulating organism that can be easily multiplied, it should be possible to develop a renewable primary source of this oil containing acetylenic fatty acids for industrial uses.

Prior art

The formation of acetylenic bonds in fatty acids in mosses occurs via the subtraction of hydrogens from a double bond (Kohn et al., 1994)

Crepis species have seed oils with high contents of acetylenic acids (Badami & Patil, 1981; Hirsinger, 1991).

Summary of the invention

The present invention provides a new method of producing acetylenic fatty acids from transgenic oil accumulating organisms.

The inventors have characterized an enzyme (acetylenase) that is responsible for the production of 9-octadecen-12-ynoic acid (crepenynic acid) from 9,12-octadecadienoic acid (linoleic acid) in membrane fractions from developing *Crepis alpina* seeds. The characterization of the acetylenase from *Crepis alpina* revealed that the acetylenase had very similar biochemical properties to the non-heme containing monooxygenases oleate delta 12 and linoleate delta 15 (omega 3) desaturases. Based on the premise that the biochemical similarities observed between the acetylenase and the enzymes producing linoleic and linolenic acid (delta 12 and delta 15 desaturases) would also be associated with similarity in the

primary sequence of these proteins a full length cDNA (pCrep1), encoding a putative acetylenase, was isolated from *Crepis alpina*.

Initially, two types of cDNA fragments, obtained by using PCR and primers designed by aligning protein sequences of delta 12 desaturases, were characterised from *C. alpina*. DNA sequence analysis revealed that one was highly homologous to all the other plant endoplasmic reticular (ER) delta 12 desaturases and the castor bean hydroxylase. The other cDNA fragment characterised had a sequence that was homologous to the ER delta 12 desaturase sequences of plants but was divergent not only in a number of non-conserved amino residues but also in a number of amino acid residues that were highly conserved in all delta 12 ER desaturases. Using northern blot analysis the gene encoding this cDNA (pCrep1) was observed to be highly expressed only in a seed specific manner when compared to expression in leaf tissue. Taken together these findings, and a consideration of the unique biochemical nature of an cell in a oilseed, provided strong evidence that the isolated cDNA (pCrep1) from *C. alpina* encode an enzyme responsible for converting linoleic acid into crepenynic acid.

Finally, conclusive evidence that the cDNA, pCrep1, from *C. alpina* encoded a plant acetylenase enzyme was obtained by the expression of this gene in yeast. The expression of this gene together with the addition of linoleic acid when culturing these yeast resulted in the production of a delta 12 acetylenic acid, 9-octadecen-12-ynoic acid (crepenynic acid), as confirmed by mass spectrometric analysis of extracted yeast fatty acids.

Therefore, in a first aspect, the present invention relates to a method of producing acetylenic compounds, characterized in that a double bond is converted to an acetylenic bond by an acetylenase.

In a preferred embodiment of the method, the acetylenic fatty acids are produced by conversion of unsaturated fatty acids to acetylenic fatty acids by a fatty acid acetylenase.

In a second aspect, the invention relates to cDNA coding for acetylenase of the mixed function monoxygenase type containing three conserved histidin motifs ($HX_{(3\ or\ 4)}H$, $HX_{(2\ or\ 3)}HH$, and $HX_{(2\ or\ 3)}HH$) according to Fig. 1 of the accompanying drawings.

In a further embodiment the invention relates to a cDNA encoding fatty acid acetylenase, such as *Crepis alpina* delta 12 acetylenase comprising the sequence according to Fig. 3 of the accompanying drawings or any nucleotide seqiences essentially homologous therewith.

A third aspect of the invention concerns use of the above described cDNA for transforming organisms. The organisms may be acetylenic compound accumulating organisms or oil accumulating organisms, respectively.

In a fourth aspect, the invention relates to organisms transformed with a acetylenase cDNA as described above. The organisms are acetylenic compound or oil accumulating, examples of the latter being oil crops, oleogeneous yeasts and moulds.

In a fifth aspect, the invention concerns acetylenic componds accumulated in organisms described above.

In a sixth aspect, the invention concerns oils from oil accumulating organisms described above.

In a preferred embodiment, the present invention relates to transforming oil accumulating organisms with the said isolated cDNA from *Crepis alpina* seed cDNA library for the purpose of producing acetylenic fatty acids acids and in particular 9-octadecen-12-ynoic acid (crepenynic acid).

D tail d description of th inventi n

C. alpina seed oil is rich in crepenynic acid [9-octadecen-12-ynoic acid (Hirsinger, 1989)]. The inventors have studied the biosynthesis of crepenynic acid in *C. alpina* seeds. The feeding of exogenous 1-^{14}C-labelled free fatty acids to intact developing cotyledons of *C. alpina* seeds demonstrated that linoleate is a precursor to crepenynic acid. This is contradictory to previous published results for the biosynthesis of crepenynic acid in *Crepis rubra* (Haigh & James, 1967). Although the reaction of acetylenic acid formation in mosses has been shown to be a desaturation process (Kohn et al.1994), such desaturation processes can be carried out by a variety of different unrelated types of plant enzymes, such as phytoene desaturases (Wieland et al. 1994) or non-heme containing proteins, the latter a class of enzymes of which some show very little amino acid sequence homologies except for three conserved histidin motifs (Shanklin et al. 1994). It has been suggested that the biosynthesis of acetylenic fatty acids occur by a sequence of intermediates catalyzed by separate enzymatic reactions. For example, acetylenic bonds were thought to be formed as a side pathway of saturated fatty acid synthesis (Diedrich & Henschel, 1991); or via an epoxygenation of a double bond with subsequent conversion to a diol which in its turn is dehydrated in two steps in order to form an acetylenic bond (Van de Loo et al, 1993). Given these conflicting alternatives the nature of an acetylenase enzyme and its mechanism of action was not known at all nor obvious at the time of the present priority patent application SE 9601236-4.

The enzyme, according to this invention, responsible for the synthesis of crepenynic acid (called the delta 12 acetylenase), was shown by the inventors to remain only active in membrane (microsomal) fractions prepared from developing seeds of *Crepis alpina*, provided that the homogenization buffer contain NADH or NADPH, catalase and free coenzyme A. The char-

acterisation of the microsomal acetylenase and its comparison with the delta 12 desaturase (responsible for the desaturation of oleate to linoleate) revealed that these enzymes had very similar properties. Both enzymes required O_2 and NADH or NADPH; where both coreductants worked equally well with both enzymes. Cyanide (CN-) and antibodies against cauliflower cytochrome b_5 inhibited both these enzymes whereas carbonmonoxide had no significant effect on either enzyme activity. These data suggested that both enzymes were biochemically similar. The oleate delta 12 hydroxylase from castor bean was also shown to have similar biochemical properties to the delta 12 desaturase despite catalyzing a different reaction (Bafor et al., 1991, Smith et al, 1992). The castor bean delta 12 hydroxylase gene was later shown to have significant sequence homology to the ER delta 12 desaturase genes (FAD 2 genes) (Van de Loo et al., 1995). Because the delta 12 acetylenase, like the delta-12 desaturase (FAD2), catalyzes a dehydrogenation between carbons 12 and 13 of an acyl chain, and like the delta 15 desaturase (FAD3) utilized linoleic acid as substrate the inventors considered the possibility that the acetylenase gene should have some sequence homology to the FAD2 and/or the FAD3 genes.

The invention will now be described more closely below in relation to the accompanying drawings and an Experimental Part.

The drawings show:

Fig. 1. Restriction map of pCrep1

Fig. 2. Restriction map of pVT-Crep1

Fig. 3. Superimposed single ion chromatograms of ions 333, 365, 367 from FADEA prepared from total fatty acids extracted from yeast strain YN94-1 transformed with pVT-Crep1. The letters denotes peaks representing the following diethylamide de-

rivatives of fatty acids: A, eicosanoic acid; B, eicosaenoic acid; C, 9-octadecen-12-ynoic acid.

Fig. 4. Superimposed single ion chromatograms of ions 333, 365, 367 from FADEA prepared from total fatty acids extracted from yeast strain YN94-1 transformed with empty vector (pVT100U; control) . The letters denotes peaks representing the following diethylamide derivatives of fatty acids: A, eicosanoic acid; B, eicosaenoic acid.

Fig. 5. A total ion chromatogramme of FADEA prepared from fatty acids enriched in the putative 9-octadecen-12 ynoic acid originating from lipid extracts of YN94-1 transformed with pVT-Crep1. The letters denotes peaks representing the following diethylamide derivatives of fatty acids: A, hecadecanoic acid; B, octadecaonoic acid; C, octadeca-9,12-dienoic acid; D. 9-octadecen-12-ynoic acid.

Fig. 6. Mass spectrum of compound corresponding to peak D in Fig.5.

EXPERIMENTAL PART

Cloning of putative acetylenase gene

An alignment of amino acid sequences from different species showed that the membrane bound fatty acid desaturases could be grouped according to the homology of their putative mature protein into three distinct groups (plastid delta 12 desaturases, ER delta 12 desaturases and delta 15 desaturases; see Sequence Listing 1). The castor bean hydroxylase (Van de Loo et al 1995) shared a high homology with the ER delta 12 desaturases to the degree that it was not easily distinguishable from these sequences. Furthermore, the sequences from all three classes of enzymes showed some degree of sequence homo logy with each other.

Based on this alignment oligonucleotide primers were designed and synthesised for these three groups of sequences and for a consensus of all of these sequences. The sequence of these primers are given below.

(i) consensus primers (primers designed to a consensus of all three groups of membrane-bound desaturases and the castor bean fatty acid hydroxylase):
sense is GSN CAY GAN TGY GSN CAY
antisense is RAN ADR TGR TGN RBN AYR TG.

(ii) plastid delta 12 desaturase primers:
sense is TGG MGN TTY AAR CAY GAY MG
antisense is GTN SWC ATC CAR AAR TGR TA.

(iii) ER delta 12 desaturase primers including the castor bean fatty acid hydroxylase:
sense is CAY GAR TGY GGN CAY CAY GC
antisense is CCN CKN ARC CAR TCC CAY TC.

(iv) delta 15 desaturase primers:
sense is ACN CAY CAY CARAAY CAY GG
antisense is CAY TGY TTN CCN CKR TAC CA.

Poly A+ RNA was isolated from developing seeds (100 mg) of *C. alpina* using a QuickPrep Micro mRNA purifcation kit from Pharmacia Biotech. All of the poly A+ RNA from this purification was precipitated and used to synthesise first strand cDNA which was primed with both oligo dT and random hexamers and synthesised with Superscript II reverse transcriptase from Gibco BRL. The polymerase chain reaction (PCR) was then used, with the described primers and this cDNA, to amplify products with the following cycling conditions:1 cycle of 94°C for 2 min, 30 cycles of (94°C, 30 sec; 50°C, 30 sec; 72°C, 30 sec) and finally one cycle of 72°C for 5 min.

Products were obtained for all the primers used; particularly noticeable was that the primers against the ER delta 12 desaturases gave significantly more product than from the other primers used. The sizes of the PCR products from the delta 12 and delta 15 primers corresponded to the sizes anticipated.

The PCR products obtained by amplification with the ER delta 12 primers and delta 15 primers were made blunt ended with T4 and klenow polymerases and cloned into the EcoRV site of the plasmid vector Bluescript. DNA sequencing of a number of the clones revealed that at least three distinct sequences had been amplified when using these two sets of primers: (i) a highly conserved delta 15 desaturase sequence (ii), a highly conserved ER delta 12 sequence and (iii) a sequence (D12V) having homology to the ER delta 12 sequences but showing distinct differences even in some amino acid residues that were highly conserved amongst all the other desaturase sequences.

The analysis of fatty acids from *C. alpina* had indicated that the crepenynic acid was probably present only in seeds. Northern blot analysis at high stringency indicated that the mRNA from the D12V sequence described above was expressed highly in seeds but not in leaves which is consistent with the observation that crepenynic acid was only observed in seeds.

A cDNA library was made from developing seeds from *C. alpina* using a Uni-ZAP XR cloning kit for cDNA from Stratagene and screened with the random labelled D12V sequence. From this screening it was estimated that cDNAs encoding the D12V sequences were highly abundant; further emphasing the high level of expression of this gene. After the isolation of single hybridising Lambda plaques, pBluescript phagemid was excised using the ExAssist/SOLR system from Stratagene. Phagemids obtained by this were subsequently used to produce double stranded DNA plasmid. From these colonies a full length clone (pCrep1, see Fig. 1) was isolated by using DNA sequencing and restriction mapping of isolated plasmid. The insert

from pCrep1, a 1.5 kb insert contained in the vector pBluescript SK, was sequenced and from this an open reading frame deduced coding for a 375 aa long protein (Sequence Listing 2). The analysis of this protein sequence revealed approximately 60% identity and 80% similarity with other plant delta 12 desaturase proteins and had noticeable differences in homology, where, certain residues that were conserved amongst all other desaturases were not in this sequence (see Sequence Listing 1). Three histidin motifs were present which have been shown to be conserved in a number of non-heme containing monoxygenases catalyzing hydroxylation and desaturation reactions (Shanklin et al. 1994).

Expression of the pCrep cDNA and detection of crepenynic acid in transgenic yeast

The pCrep1 open reading frame was released from pCrep1 on a *Sma*I/*Xho*I restriction fragment and the 1.5 kb *Crep1* open reading frame recovered by gelpurification (Langridge et al., 1980). pVT100-U DNA (Vernet et al., 1987) was digested using *Pvu*II and *Xho*I. 50 ng *Pvu*II/*Xho*I-linearized pVT100 was ligated with 100 ng 1.5 kb *Sma*I/*Xho*I fragment corresponding to the Crep1 open reading frame using T4 DNA ligase (NBL Genen Science Ltd., UK). Part of the ligation mixture was used to transform competent *E.coli* DHa cells. One clone (pVT-Crep1), which contained the expected 1.5 kb insert, was chosen and the contruct checked by digestion with *Eco*RI, or *Hind*III + *Xba*I. Both digests gave the expected products (approx. 5.3, 2.3 and 0.8 kb for the EcoRI digest, and release of the 1.5 kb open reading frame with the *Hind*III + *Xba*I digest). pVT-Crep1 DNA (see Fig. 2), or empty vector pVT100U, was used to transform *Saccharomyces cerevisiae* strains YN94-1 and C13-ABYS86, using the PLATE method of Elble (1992). Overnight yeast transformants were spread on SCD minus uracil agar and single colonies were streaked onto fresh selective (minus uracil) plates.

The YN94-1 and C13-ABYS86 strains of yeast transformed with pVT-Crep1 DNA and with empty vector (pVT100U; control) were cultivated in shaking cultures at 28°C for five hours in selective media (without uracil; 400 ml) whereafter 40 ml of cultivation media containing linoleic acid dispersed in Tween 40® was added to the culture to give a final concentration of 0.03% linoleic acid and 1% Tween 40® (w/w). After cultivation for an additional 78h at 28°C the cells were pelleted by centrifugation and washed by dispersion in 20 ml of 0.1M Tris-HCl buffer pH. 7.8 containing 1% Tween 40® and repelleted by centrifugation.The cells were further washed by resuspension in 20 ml of 0.1M Tris-HCl buffer pH. 7.8 and pelleted again. The cells were thereafter extracted in a mixture of chloroform/methanol/ 0.15M acetic acid (1:2:0.8 by vol.) in a Braun MSK glass bead cell homogenizer (B. Braun Biotech International, Melsungen, Germany) at 4000 r.p.m. for 20 s. The yeast lipids were extracted from the mixture into a chloroform phase by adding chloroform and 0.15M acetic acid to yield final proportions of 1:1:0.9 (by vol.) of chloroform, methanol and 0.15 M acetic acid . After centrifugation of the mixture the lipid containing chloroform phase was removed and evaporated to dryness under a stream of nitrogen.

The lipohilic residue were methylated with methanolic HCl (4% w/w) at 85°C for 45 min wherafter the fatty acid methyl esters were extracted into n-hexane. Gas liquid (GC) chromatogrammes of the methyl esters separated on a glass column (2.5m x 3 mm i.d.) containing 3% SP-2300 on Supelcoport 100/120 mesh (Supelco, Bellefonte, P. USA) revealed a peak with the same retention time as authentic 9-octadecen-12 ynoic acid metyl ester constituting up to 0.3% of total peak areas in samples prepared from yeast transformed with pVT-Crep1 but not in samples prepared from yeast transformed with empty vector (pVT100U; control).

Since acetylenic fatty acid methyl esters can be partially separated from other fatty acid methylesters on silica gel

thin layer chromatography, the methylesters prepared from YN94-1 transformed with pVT-Crep1 were separated on silica gel 60 thin layer chromatography plates (Merck, Darmstadt, Germany) by developing the plate in hexane/diethyl ether/acetic acid (85:15:1 by vol.). An area located just below the main methyl ester area was removed from the plate and the lipids were eluted with methanol/chloroform (2:1) and analyzed by gas liquid chromatography. The fraction were shown to consist of fatty acid methylesters where the peak with the same retention time as 9-octadecen-12 ynoic acid metyl ester made up 12.5% of the total peak area.

The methyl ester fraction enriched in the putative 9-octadecen-12 ynoic acid methyl ester as well as total fatty acid methyl esters prepared from YN94-1 transformed with pVT-Crep1 and YN94-1 transformed with empty vector (pVT100U; control) were hydrolyzed in 2.5M KOH in aqueous methanol (15% methanol, by vol.) at 90°C for 1 h. The free fatty acids were extracted into hexane after acidicifiction with HCl and the hexane phase was evaporated to dryness under a stream of nitrogen.

Fatty acid diethylamides (FADEA) were prepared from the free fatty acids according to Nilsson and Liljenberg (1991). The FADEA were either injected directly on a gas liquid chromatography coupled to mass spectrometer (GC-MS) or subjected to further purification by silica gel thin layer chromatography by developing the plate in heptane/diethyleter/acteic acid (50:50:1, by vol.).

The FADEA were analyzed on a Hewlett-Packard 5890 II gas chromatograph equipped with a DB225 (0.25 mm i.d. x 30 m, J&W, Folsom, USA) in series with a Rtx 2330 (Restek Corp., PA, USA) fused silica capillary column, coupled with a Hewlett-Packard 5989A mass spectrometer working in electron impact mode at 70 eV. Injection technique was cold splitless at 100°C and then the temperature was raised as quickly as possible to 240°C.

Oven temperature was 100°C for 7 min, then 20°C per min to 190°C and then 1°C per min to a final temp. of 225°C where it was kept for another 20 min. The double bond positions were determined according to Nilsson and Liljenberg (1991).

Single ion chromatogrammes of masses corresponding to the molecular ion of FADEA prepared from total fatty acids from YN94-1 transformed with pVT-Crep1 and from YN94-1 transformed with empty vector (pVT100U; control) are shown in Fig.5 and Fig.6, respectively. Chromatogram of FADEA from YN94-1 transformed with pVT-Crep1 showed a peak of mass 333 (corresponding to the molecular weigth of 9-octadecen-12 ynoic acid diethylamide) which was absent in the chromatogram of FADEA from YN94-1 transformed with empty vector (pVT100U; control). The peak had a retention time of 57.3 min and was located between peaks corresponding to eicosanoic and eicosenoic FADEA derivatives.

A total ion chromatogramme of FADEA prepared from fatty acids enriched in the putative 9-octadecen-12 ynoic acid by thin layer chromatography (as described above) originating from lipid extracts of YN94-1 transformed with pVT-Crep1 is shown in Fig. 5. Mass spectrum (Fig.6) of the putative 9-octadecen-12 ynoic acid diethylamide derivative (peak D in Fig.5) showed a gap in mass of 26 amu instead of regular 28 between carbon 7 and 9 indicating a double bond at position 9. Further more there was a gap of 24 amu instead of regular 28 between carbon atom 10 and 12 indicating acetylenic bond at position 12. The peak D produced a mass spectrum identical to that of authentic 9-octadecen-12 ynoic acid diethylamide prepared from oils from *Crepis alpina* seeds. Thus the peak D in the chromatogram in Fig 5 was unambigously identified as 9-octadecen-12 ynoic acid diethylamide derivative. Since the compound was absent in yeast strains not transformed with the Crep1 cDNA it is clear that the Crep1 cDNA codes for a delta-12 fatty acid acetylenase.

REFERENCES

Badami, R.C., and Patil, K.B. (1981). Strucure and occurrence of unusual fatty acids in minor seed oils. Progress in Lipid Research, 19, 119-53.

Banas, A., Bafor, M., Wiberg, E., Lenman, M., Ståhl, U. and Stymne, S. (1997) Biosynthesis of an acetylenic fatty acid in microsomal preparations from developing seeds of *Crepis alpina*. In: Williams, J.P., Mobasher, K.U., Lem, N.W. (eds) Physiology, biochemistry and molecular biology of plant lipids. Kluwer Academic Publisher, Dordrecht. *In-press*

Birnboim, H.C., and Doly, J. (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7, 1513-1523

Diedrich, M. & Henschel, K.P. (1991) The natural occurence of unusual fatty acids: Part 3. Acetylenic fatty acids. Nahrung 35, 193-202

Elble, R. (1992) A simple and efficient procedure for transformation of yeasts. Biotechniques 13, 18-20

Haigh, W.G. & James, A.T. Biochim. Biophys. Acta 137, 391-392

Hirsinger, F. (1989). New annual oil crops. In Oil crops of the world, (eds. G. Röbbelen, R.K. Downey and A. Ashri), pp.518-532. Mc-Graw-Hill Inc.

Kohn, G., Hartmann, E., Stymne, S. & Beutelmann, P. (1994) Biosynthesis of acetylenic acids in the moss Ceratodon purpureus. J. Plant Physiol. 144, 265-271

Langridge, J., Langridge, P and Bergquist P.L. (1980) Extraction of nucleic acids from agarose gels. Anal. Biochem. 103, 264-271

Nilsson, R. and Liljenberg, C. (1991) The determination of double bond positions in polyunsaturated fatty acids - Gaschromatography/ mass spectrometry of the diethylamide derivative. Phytochemical analysis 2, 253-259

Shanklin, J., Whittle, E. & Fox, B.G. (1994) Eight histidine residues are catalytically essential in membrane -associated iron enzyme, stearoyl-CoA desaturase and are conserved in alkane hydroxylase and xylene monoxygenase. Biochemistry 33, 12787-12794.

Stymne, S. & Lenman, M. (1996) Novel plant enzyme and use thereof. Swedish patent application no. 9601236-4, 96-03-29.

Vernet, T., Dignard, D. and Thomas, D.Y. (1987) A family of yeast expression vectors containing the phafe f1 intergenic region. Gene 52, 225-233

Wieland, B., Feil, C., Gloria-Maercker, E., Thumm, G., Lechner, M., Bravo, J.M., Poralla, K. & Goetz, F. (1994) Genetic and biochemical analysis of the biosynthesis of the yellow carotenoid 4,4'-diaponeurosporene of Staphylococcus aureus. J. Bacteriol. 176, 7719-7726.

van de Loo, F.J., Broun, P., Turner, S. & Somerville, C. (1995) An oleate D12-hydroxylase from Ricinus communis L. is a fatty acid acyl desaturase homolog. Proc. Natl. Acad. Sci. USA 95, 6743-6747.

van de Loo, F.J., Fox, G. & Somerville, C. (1993) Unusual fatty acids. In: T.S. Moore
Jr (ed.) Lipid Metabolism in Plants. CRC Press, Boca Raton , page 105.

INFORMATION ABOUT SEQUENCE LISTING NO 1

Alignment of amino acid sequences from delta 12 ER and plastid desaturases, delta 15 desaturases and from the castor bean hydroxylase. Also included in this alignment is the protein sequence derived from pCrep1 (crepis). Underlined are three histidin motifs that are conserved in non-heme containing monoxygenases.

Sequences given in this alignment together with their accession numbers are : bnom6des.seq, delta 12 desaturase from *Brassica napus* (L29214);
gmom6des.seq, delta 12 desaturase from *Glycine max* (L29215);
atom3des.seq, delta15 desaturase from *Arabidopsis thaliana* (L22961);
bnom3des.seq, delta 15 from *Brassica napus* (L22963);
rcom3des.seq, delta 15 desaturase from *Ricinus communis* (L25897);
siom3des.seq, delta 15 desaturase from oriental sesame (U25817);
ldd15des.seq, delta 15 desaturase from *Limnanthes douglasii* (U17063);
gsom3des, delta 15 desaturase from *Glycine max* (L22965);
atom3bdes.seq, delta15 desaturase from *Arabidopsis thaliana* (D17579);
bnom31des.seq, delta 15 from *Brassica napus* (L22962);
gsom3bdes.seq, delta 15 desaturase from *Glycine max* (L22964);
atd12des.seq, delta12 desaturase from *Arabidopsis thaliana* (L26296);
gmom6bdes.seq delta 12 desaturase from *Glycine max* (L43921);
scom12des.seq, delta 12 desaturase from *S. commersonii* (X92847);
gmom6ades.seq, delta 12 desaturase from *Glycine max* (L43920);
rchyd.seq, oleate 12-hydroxylase from *Ricinus communis* (U22378);
crepis, *Crepis alpina* acetylenase from this document.

SEQUENCE LISTING 1

```
                1                                                   50
 bnom6des.seq   .......... .......... ....MASRIA DSLFAFTGPQ QCLPRAPKLA
 gmom6des.seq   .......... .......... ....MACTLA DSLLLFKGSY Q.KPVLRRDI
 atom3des.seq   .MANLVLSEC GIRPLPRIYT TPRSNFLSNN N...KFRPSL SSSSYKTSSS
 bnom3des.seq   .......... .......... .......... .......... ..........
 rcom3des.seq   MAAGWVLSEC GLRPLPRIYS RPRIGFTSKT TNLLKLRELP DSKSYNLCSS
 siom3des.seq   .MASWVLSEC GLRPLPRVYP KPRTGHPLLN SNPTKLRFSR TDLGNGSS..
 ldd15des.seq   .MASWVLSQY ALNPLPHIFR TPRTSITSHK .........L TVSHTNNRAT
 gsom3des.seq   .MATWYHQKC GLKPLAPVIP RPRTGAALSS TSRVEF.... ..LDTNKVVA
atom3bdes.seq   .......... .......... .......... .......... ..........
bnom31des.seq   .......... .......... .......... .......... ..........
gsom3bdes.seq   .......... .......... .......... .......... ..........
 atd12des.seq   .......... .......... .......... .......... ..........
gmom6bdes.seq   .......... .......... .......... .......... ..........
scom12des.seq   .......... .......... .......... .......... ..........
gmom6ades.seq   .......... .......... .......... .......... ..........
    rchyd.seq   .......... .......... .......... .......... ..........
       crepis   .......... .......... .......... .......... ..........

                51                                                  100
 bnom6des.seq   SARLSPGVYA VRPIDLLLKG TRRTFLVPAK KRIGCIKAVF VPVAPPSADN
 gmom6des.seq   AARYSPGIFS LNSNGLIQKR FRRQRNFVTR NKVTVIHAVA IPVQPAPVES
 atom3des.seq   PLSFGLNSRD GFTRNWALNV STPLTTPIFE ESP....... ....LEEDNK
 bnom3des.seq   .......... .......... .......... .......... ..........
 rcom3des.seq   FKVSSWSNSK QSNWALNVAV PVNVSTVSGE DDREREEFNG IVN.VDEGKG
 siom3des.seq   ...FCLSSGI LREKNWALRV SAPLRVLQVE EEEENKEGER VIN.GGEE..
 ldd15des.seq   PDLTKLSLIK FRERKLGLRV SAPFQIASTT PE........ .....EEDEV
 gsom3des.seq   GPKFQPLRCN LRERNWGLKV SAPLRVASIE EEQKSVDLTN GTNGVEHEKL
atom3bdes.seq   .......... .......... .......MVV AMDQRTNVNG DPGAGDRKKE
bnom31des.seq   .......... .......... .......MVV AMDQRSNANG D.........
gsom3bdes.seq   .......... .......... ........MV KDTKPLAYAA NNGYQQKGSS
 atd12des.seq   .......... .......... ......MGAG GRMPVP.... TSSKKSETDT
gmom6bdes.seq   .......... .......... ......MGAG GRTDVP.... PANRKSEVDP
scom12des.seq   .......... .......... ......MGAG GRMSAP.... NGETEVKRNP
gmom6ades.seq   .......... .......... MGLAKETTMG GRGRVA.... KVEVQGK.KP
    rchyd.seq   .......... .......... ......MGGG GRMSTVITSN NSEKKGGSSH
       crepis   .......... .......... ......MGGG GR........ ..GRTSQKPL

                101                                                 150
 bnom6des.seq   AEDREQLAES YGFKQIGQDL PDNVTLKDIM DTLPKEVFEI DDVKAWKSVL
 gmom6des.seq   AEYRKQLAED YGFRQVGEPL SDDVTLKDVI NPLPKEVFEI DDVKAWKSVL
 atom3des.seq   QRFDPGAPPP FNLADIRAAI PKHCWVKNPW KSLSYVVRDV AIVFA.....
 bnom3des.seq   .......... .......... .......... ..MSYVVREL AIVFA.....
 rcom3des.seq   EFFDAGAPPP FTLADIRAAI PKHCWVKNPW RSMSYVLRDV VVVFG.....
 siom3des.seq   ..FDPGAPPP FKLSDIREAI PKHCWVKDPW RSMGYVVRDV AVVFG.....
 ldd15des.seq   AEFDPGSPPP FKLADIRAAI PKHCWVKNQW RSMSYVVRDV VIVLG.....
 gsom3des.seq   PEFDPGAPPP FNLADIRAAI PKHCWVKDPW RSMSYVVRDV IAVFG.....
atom3bdes.seq   ERFDPSAQPP FKIGDIRAAI PKHCWVKSPL RSMSYVVRDI IAVAA.....
bnom31des.seq   ERFDPSAQPP FKIGDIRAAI PKHCWVKSPL RSMSYVARDI FAVVA.....
gsom3bdes.seq   FDFDPSAPPP FKIAEIRASI PKHCWVKNPW RSLSYVLRDV LVIAA.....
 atd12des.seq   TKRVPCEKPP FSVGDLKKAI PPHCFKRSIP RSFSYLISDI IIASC.....
gmom6bdes.seq   LKRVPFEKPQ FSLSQIKKAI PPHCFQRSVL RSFSYVVYDL TIAFC.....
scom12des.seq   LQKVPTSKPP FTVGDIKKAI PPHCFQRSLI RSFSYVVYDL ILVSI.....
gmom6ades.seq   LSRVPNTKPP FTVGQLKKAI PPHCFQRSLL TSFSYVVYDL SFAF......
    rchyd.seq   LKRAPHTKPP FTLGDLKRAI PPHCFERSFV RSFSYVAYDV CLSFL.....
       crepis   MERVSVD.PP FTVSDLKQAI PPHCFKRSVI RSSYYIVHDA IIAYI.....
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SEQUENCE LISTING 1 (cont.)

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                151                                                 200
 bnom6des.seq   ISVTSYALGL FMIAKAPWYL LPLAWAWTGT AVTGFFVIGH DCAHKSFSKN
 gmom6des.seq   ISVTSYALGL FMISKAPWYL LPLAWVWTGT AITGFFVIGH DCAHRSFSSN
 atom3des.seq   LAAGAAYL.. .....NNWIV WPLYWLAQGT MFWALFVLGH DCGHGSFSND
 bnom3des.seq   LAAGAAYL.. .....NNWLV WPLYWIAQGT MFWALFVLGH DCGHGSFSND
 rcom3des.seq   LAAVAAYF.. .....NNWVA WPLYWFCQGT MFWALFVLGH DCGHGSFSNN
 siom3des.seq   LAAVAAYF.. .....NNWVV WPLYWFAQST MFWALFVLGH DCGHGSFSND
 ldd15des.seq   LAAAAVAA.. .....NSWAV WPLYWVAQGT MFWALFVLGH DCGHGSFSNN
 gsom3des.seq   LAAAAAYL.. .....NNWLV WPLYWAAQGT MFWALFVLGH DCGHGSFSNN
atom3bdes.seq   LAIAAVYV.. .....DSWFL WPLYWAAQGT LFWAIFVLGH DCGHGSFSDI
bnom31des.seq   LAVAAVYF.. .....DSWFF WPLYWAAQGT LFWAIFVLGH DCGHGSFSDI
gsom3bdes.seq   LVAAAIHF.. .....DNWLL WLIYCPIQGT MFWALFVLGH DCGHGSFSDS
 atd12des.seq   FYYVATNYFS LLPQPLSYLA WPLYWACQGC VLTGIWVIAH ECGHHAFSDY
gmom6bdes.seq   LYYVATHYFH LLPGPLSFRG MAIYWAVQGC ILTGVWVIAH ECGHHAFSDY
scom12des.seq   MYYVANTYFH LLPSPYCYIA WPIYWICQGC VCTGIWVNAH ECGHHAFSDY
gmom6ades.seq   IFYIATTYFH LLPQPFSLIA WPIYWVLQGC LLTGVWVIAH ECGHHAFSKY
     rchyd.seq  FYSIATNFFP YISSPLSYVA WLVYWLFQGC ILTGLWVIGH ECGHHAFSEY
        crepis  FYFLADKYIP ILPAPLAYLA WPLYWFCQAS ILTGLWVIGH ECGHHAFSDY

                201                                                 250
 bnom6des.seq   KLVEDIVGTL AFLPLVYPYE PWRFKHDRHH AKTNMLVHDT AWQPVPPEEF
 gmom6des.seq   KLVEDIVGTL AFMPLIYPYE PWRFKHDRHH AKTNMLREDT AWHPVWKDEF
 atom3des.seq   PKLNSVVGHL LHSSILVPYH GWRISHRTHH QNHGHVENDE SWHPMSEKIY
 bnom3des.seq   PRLNSVVGHL LHSSILVPYH GWRISHRTHH QNHGHVENDE SWHPMSEKIY
 rcom3des.seq   PKLNSVVGHL LHSSILVPYH GWRISHRTHH QNHGHVENDE SWHPLSEKIF
 siom3des.seq   PKLNSVVGHI LHSSILVPYH GWRISHRTHH QNHGHVENDE SWHPLSEKIY
 ldd15des.seq   HKLNSVVGHL LHSSILVPYH GWRIRHRTHH QNHGHVENDE SWHPMSEKLF
 gsom3des.seq   SKLNSVVGHL LHSSILVPYH GWRISHRTHH QHHGHAENDE SWHPLPEKLF
atom3bdes.seq   PLLNSVVGHI LHSFILVPYH GWRISHRTHH QNHGHVENDE SWVPLPERVY
bnom31des.seq   PLLNTAVGHI LHSFILVPYH GWRISHRTHH QNHGHVENDE SWVPLPEKLY
gsom3bdes.seq   PLLNSLVGHI LHSSILVPYH GWRISHRTHH QNHGHIEKDE SWVPLTEKIY
 atd12des.seq   QWLDDTVGLI FHSFLLVPYF SWKYSHRRHH SNTGSLERDE VFVPKQKSAI
gmom6bdes.seq   QLLDDIVGLI LHSALLVPYF SWKYSHRRHH SNTGSLERDE VFVPKQKSCI
scom12des.seq   QWVDDTVGLI LHSALLVPYF SWKYSHRRHH SNTGSLERDE VFVPKPKSQL
gmom6ades.seq   QWVDDVVGLT LHSTLLVPYF SWKISHRRHH SNTGSLDRDE VFVPKPKSKV
     rchyd.seq  QLADDIVGLI VHSALLVPYF SWKYSHRRHH SNIGSLERDE VFVPKSKSKI
        crepis  QWVDDTVGFI LHSFLMTPYF SWKYSHRNHH ANTNSLDNDE VYIPKSKAKV

                251                                                 300
 bnom6des.seq   DS........ .SPVLRKAII FGYGPIRPWL SI......AH WVNWHFNLRK
 gmom6des.seq   ES........ .TPLLRKAII YGYGPFRCWM SI......AH WLMWHFDLKK
 atom3des.seq   NTLDK..... PTRFFRFTLP LVMLAYPFYL WARSPGKK.. ..GSHYHPDS
 bnom3des.seq   KSLDK..... PTRFFRFTLP LVMLAYPFYL WARSPGKK.. ..GSHYHPDS
 rcom3des.seq   KSLDN..... VTKTLRFSLP FPMLAYPFYL WSRSPGKK.. ..GSHFHPDS
 siom3des.seq   KNLDT..... ATKKLRFTLP FPLLAYPIYL WSRSPGKQ.. ..GSHFHPDS
 ldd15des.seq   RSLDK..... IALTFRFKAP FPMLAYPFYL WERSPGKT.. ..GSHYHPDS
 gsom3des.seq   RSLDT..... VTRMLRFTAP FPLLAFPVYL FSRSPGKT.. ..GSHFDPSS
atom3bdes.seq   KKLPH..... STRMLRYTVP LPMLAYPLYL CYRSPGKE.. ..GSHFNPYS
bnom31des.seq   KNLSH..... STRMLRYTVP LPMLAYPLYL WYRSPGKE.. ..GSHYNPYS
gsom3bdes.seq   KNLDS..... MTRLIRFTVP FPLFVYPIYL FSRSPGKE.. ..GSHFNPYS
 atd12des.seq   KWYGKYLNNP LGRIMMLTVQ F.VLGWPLYL AFNVSGRPYD GFACHFFPNA
gmom6bdes.seq   KWYSKYLNNP PGRVLTLAVT L.TLGWPLYL ALNVSGRPYD RFACHYDPYG
scom12des.seq   GWYSKYLNNP PGRVLSLTIT L.TLGWPLYL AFNVSGRPYD RFACHYDPYG
gmom6ades.seq   AWFSKYLNNP LGRAVSLLVT L.TIGWPMYL AFNVSGRPYD SFASHYHPYA
     rchyd.seq  SWYSKYSNNP PGRVLTLAAT L.LLGWPLYL AFNVSGRPYD RFACHYDPYG
        crepis  ALYYKVLNHP PGRLLIMFIT F.TLGFPLYL FTNISGKKYE RFANHFDPMS
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SEQUENCE LISTING 1 (cont.)

```
                301                                                      350
 bnom6des.seq   ..FRPSEVNR VKISLACVFA FMAVGWPLII YKVGVLGWVK FWLMPWLGYH
 gmom6des.seq   ..FRPSEVPR VKISLACVFA FIAIGWPLII YKTGIMGWIK FWLMPWLGYH
 atom3des.seq   DLFLPKERKD VLTSTACWTA .MAALLVCLN FTIGPIQMLK LYGIPYWINV
 bnom3des.seq   DLFLPKERND VLTSTACWTA .MAVLLVCLN FVMGPMQMLK LYVIPYWINV
 rcom3des.seq   GLFVPKERKD IITSTACWTA .MAALLVYLN FSMGPVQMLK LYGIPYWIFV
 siom3des.seq   DLFVPNEKKD VITSTVCWTA .MLALLVGLS FVIGPVQLLK LYGIPYLGNV
 ldd15des.seq   DLFVPSEKKD VITSTICWTT .MVGLLIGLS FVMGPIQILK LYVVPYWIFV
 gsom3des.seq   DLFVPNERKD VITSTACWAA .MLGLLVGLG FVMGPIQLLK LYGVPYVIFV
atom3bdes.seq   SLFAPSERKL IATSTTCWSI .MFVSLIALS FVFGPLAVLK VYGVPYIIFV
bnom31des.seq   SLFAPSERKL IATSTTCWSI .MLATLVYLS FLVGPVTVLK VYGVPYIIFV
gsom3bdes.seq   NLFPPSERKG IAISTLCWAT .MFSLLIYLS FITSPLLVLK LYGIPYWIFV
 atd12des.seq   PIYNDRERLQ IYLSDAGILA .VCFGLYRYA AAQGMASMIC LYGVPLLIVN
gmom6bdes.seq   PIYSDRERLQ IYISDAGVLA .VVYGLFRLA MAKGLAWVVC VYGVPLLVVN
scom12des.seq   PIYNNRERLQ IFISDAGVLG .VCYLLYRIA LVKGLAWLVC VYGVPLLVVN
gmom6ades.seq   PIYSNRERLL IYVSDVALFS .VTYSLYRVA TLKGLVWLLC VYGVPLLIVN
    rchyd.seq   PIFSERERLQ IYIADLGIFA .TTFVLYQAT MAKGLAWVMR IYGVPLLIVN
       crepis   PIFKERERFQ VLLSDLGLLA .VLYGVKLAV AAKGAAWVTC IYGIPVLGVF

                351                                                      400
 bnom6des.seq   FWMSTFTMVH HTAPH..IPF KPADEWNAAQ AQLNGTVHCD YPSWIEILCH
 gmom6des.seq   FWMSTFTMVH HTAPY..IPF KYSEEWNRAQ AQLNGTVHCD YPKWIEILCH
 atom3des.seq   MWLDFVTYLH HHGHEDKLPW YRGKEWSYLR GGL.TTLDRD YGLINNIHHD
 bnom3des.seq   MWLDFVTYLH HHGHEDKLPW YRGKEWSYLR GGL.TTLDRD YGLINNIHHD
 rcom3des.seq   MWLDFVTYLH HHGHEDKLPW YRGKAWSYLR GGL.TTLDRD YGWINNIHHD
 siom3des.seq   MWLDLVTYLH HHGHEDKLPW YRGKEWSYLR GGL.TTLDRD YGWINNIHHD
 ldd15des.seq   MWLDFVTYLD HHGHEDKLPW YRGEEWSYLR GGL.TTLDRD YGLINNIHHD
 gsom3des.seq   MWLDLVTYLH HHGHEDKLPW YRGKEWSYLR GGL.TTLDRD YGWINNIHHD
atom3bdes.seq   MWLDAVTYLH HHGHDEKLPW YRGKEWSYLR GGL.TTIDRD YGIFNNIHHD
bnom31des.seq   MWLDAVTYLH HHGHDDKLPW YRGKEWSYLR GGL.TTIDRD YGIFNNIHHD
gsom3bdes.seq   MWLDFVTYLH HHGHHQKLPW YRGKEWSYLR GGL.TTVDRD YGWIYNIHHD
 atd12des.seq   AFLVLITYLQ H..THPSLPH YDSSEWDWLR GAL.ATVDRD YGILNKVFHN
gmom6bdes.seq   GFLVLITFLQ H..THPALPH YTSSEWDWLR GAL.ATVDRD YGILNKVFHN
scom12des.seq   GFLVLITYLQ H..THPSLPH YDSTEWDWLR GAL.ATCDRD YGVLNKVFHN
gmom6ades.seq   GFLVTITYLQ H..THFALPH YDSSEWDWLK GAL.ATMDRD YGILNKVFHH
    rchyd.seq   CFLVMITYLQ H..THPAIPR YGSSEWDWLR GAM.VTVDRD YGVLNKVFHN
       crepis   IFFDIITYLH H..THLSLPH YDSSEWNWLR GAL.STIDRD FGFLNSVLHD

                401                                                      450
 bnom6des.seq   DINVHIPHHI SPRIPSYNLR AAHQSIQENW GKYTNLATWN WRLMKTIMTV
 gmom6des.seq   DINVHIPHHI SPRIPSYNLR AAHKSLQENW GQYLNEASWN WRLMKTIMTV
 atom3des.seq   I.GTHVIHHL FPQIPHYHLV EATEAAKPVL GKYYREPDKS .GPLPLHLLE
 bnom3des.seq   I.GTHVIHHL FPQIPHYHLV EATEAAKPVL GKYYREPDKS .GPLPLHLLG
 rcom3des.seq   I.GTHVIHHL FPQIPHYHLV EATEAAKPVM GKYYREPKKS .GPLPLHLLG
 siom3des.seq   I.GTHVIHHL FPQIPHYHLI EATEAAKPVL GKYYREPKKS .APLPFHLLG
 ldd15des.seq   I.GTHVIHHL FPQIPHYHLV EATQAAKPIF GKYYKEPAKS .KPLPFHLID
 gsom3des.seq   I.GTHVIHHL FPQIPHYHLV EATEAAKPVF GKYYREPKKS AAPLPFHLIG
atom3bdes.seq   I.GTHVIHHL FPQIPHYHLV DATKAAKHVL GRYYREPKTS .GAIPIHLVE
bnom31des.seq   I.GTHVIHHL FPQIPHYHLV DATKSAKHVL GRYYREPKTS .GAIPIHLVE
gsom3bdes.seq   I.GTHVIHHL FPQIPHYHLV EATQAAKPVL GDYYREPERS .APLPFHLIK
 atd12des.seq   ITDTHVAHHL FSTMPHYNAM EATKAIKPIL GDYYQFDGTP .......WYV
gmom6bdes.seq   ITDTHVAHHL FSTMPHYHAM EATKAIKPIL GEYYRFDETP .......FVK
scom12des.seq   ITDTHVVHHL FSTMPHYNAM EATKAVKPLL GDYYQFDGTP .......IYK
gmom6ades.seq   ITDTHVAHHL FSTMPHYHAM EATNAIKPIL GEYYQFDDTP .......FYK
    rchyd.seq   IADTHVAHHL FATVPHYHAM EATKAIKPIM GEYYRYDGTP .......FYK
       crepis   VTHTHVMHHL FSYIPHYHAK EARDAINTVL GDFYKIDRTP .......ILK
```

SEQUENCE LISTING 1 (cont.)

```
                451                                                  493
 bnom6des.seq   CHVYDKEENY IPFDRLAPEE SQPITFLKKA MPDYAA..... ...
 gmom6des.seq   CQVYDKEKSL CCLRRTCP.. .......... .......... ...
 atom3des.seq   ILAKSIKEDH YV.....SDE GEVVYYKADP NLYGEVKVRA D..
 bnom3des.seq   ILAKSIKEDH FV.....SDE GDVVYYEADP NLYGEIKVTA E..
 rcom3des.seq   SLVRSMKEDH YV.....SDT GDVVYYQKDP KLSGIGGEKT E..
 siom3des.seq   DLTRSLKRDH YV.....SDV GDVVYYQTDP QLTGAEKS.. ...
 ldd15des.seq   VLLKSLKRDH FV.....PDT GDIVYYQSDP QISGSLKPE. ...
 gsom3des.seq   EIIRSFKTDH FV.....SDT GDVVYYQTDS KINGSSKLE. ...
atom3bdes.seq   SLVASIKKDH YV.....SDT GDIVFYETDP DLYVYASDKS KIN
bnom31des.seq   SLVASIKKDH YV.....SDT GDIVFYETDP DLYVYASDKS KIN
gsom3bdes.seq   YLIQSMRQDH FV.....SDT GDVVYYQTDS LLLHSQRD.. ...
 atd12des.seq   AMYREAKECI YVEPDREGDK KGVYWYNNKL .......... ...
gmom6bdes.seq   AMWREARECI YVEPDQSTES KGVFWYNNKL .......... ...
scom12des.seq   EMWREAKECL YVEKDESSQG KGVFWYKNKL .......... ...
gmom6ades.seq   ALWREARECL YVEPDEGTSE KGVYWYRNKY .......... ...
    rchyd.seq   ALWREAKECL FVEPDEGAPT QGVFWYRNKY .......... ...
       crepis   AMWREAKECI FIEPEKGRES KGVYWY.NKF .......... ...
```

SEQUENCE LISTING 2

Nucleotide sequence and derived amino acid sequence of the open reading frame from plasmid pCrepl.

```
ATGGGTGGCGGTGGCCGTGGTCGGACTTCGCAAAAACCCCTCATGGAACGTGTCTCAGTT
 M  G  G  G  G  R  G  R  T  S  Q  K  P  L  M  E  R  V  S  V

GATCCACCCTTCACCGTGAGTGATCTCAAGCAAGCAATCCCTCCCCATTGCTTCAAGCGA
 D  P  P  F  T  V  S  D  L  K  Q  A  I  P  P  H  C  F  K  R

TCTGTAATCCGTTCCTCTTACTACATAGTCCACGATGCTATTATCGCCTACATCTTCTAC
 S  V  I  R  S  S  Y  Y  I  V  H  D  A  I  I  A  Y  I  F  Y

TTCCTTGCCGACAAATACATTCCGATTCTCCCTGCCCCTCTAGCCTACCTCGCTTGGCCC
 F  L  A  D  K  Y  I  P  I  L  P  A  P  L  A  Y  L  A  W  P

CTTTACTGGTTCTGTCAAGCTAGCATCCTCACCGGCTTATGGGTCATCGGTCACGAATGC
 L  Y  W  F  C  Q  A  S  I  L  T  G  L  W  V  I  G  H  E  C

GGTCACCATGCCTTCAGCGACTACCAGTGGGTTGACGACACTGTGGGCTTCATCCTCCAC
 G  H  H  A  F  S  D  Y  Q  W  V  D  D  T  V  G  F  I  L  H

TCGTTTCTCATGACCCCGTATTTCTCCTGGAAATACAGCCACCGGAACCACCATGCCAAC
 S  F  L  M  T  P  Y  F  S  W  K  Y  S  H  R  N  H  H  A  N

ACAAATTCGCTTGACAACGATGAAGTTTACATCCCCAAAAGCAAGGCCAAAGTCGCGCTT
 T  N  S  L  D  N  D  E  V  Y  I  P  K  S  K  A  K  V  A  L

TACTATAAAGTTCTCAACCACCCACCTGGCCGACTGTTGATTATGTTCATCACCTTCACC
 Y  Y  K  V  L  N  H  P  P  G  R  L  L  I  M  F  I  T  F  T

CTAGGCTTCCCTCTATACCTCTTTACCAATATTTCCGGCAAGAAGTATGAAAGGTTTGCC
 L  G  F  P  L  Y  L  F  T  N  I  S  G  K  K  Y  E  R  F  A

AACCATTTCGACCCCATGAGTCCGATTTTCAAAGAGCGTGAGCGGTTTCAGGTCTTGCTA
 N  H  F  D  P  M  S  P  I  F  K  E  R  E  R  F  Q  V  L  L

TCGGATCTTGGCCTTCTTGCTGTGCTTTACGGAGTTAAACTTGCGGTAGCAGCGAAAGGC
 S  D  L  G  L  L  A  V  L  Y  G  V  K  L  A  V  A  A  K  G

GCCGCCTGGGTGACGTGCATTTACGGAATTCCAGTTTTAGGCGTGTTTATCTTTTTCGAT
 A  A  W  V  T  C  I  Y  G  I  P  V  L  G  V  F  I  F  F  D

ATCATCACCTACTTGCACCACACCCATCTGTCGTTGCCTCATTATGATTCATCTGAATGG
 I  I  T  Y  L  H  H  T  H  L  S  L  P  H  Y  D  S  S  E  W

AACTGGCTCAGAGGGGCTTTGTCAACAATCGATAGGGACTTTGGGTTCCTGAATAGTGTG
 N  W  L  R  G  A  L  S  T  I  D  R  D  F  G  F  L  N  S  V

CTCCATGATGTTACACACACTCACGTTATGCATCATCTGTTTTCATACATTCCACACTAT
 L  H  D  V  T  H  T  H  V  M  H  H  L  F  S  Y  I  P  H  Y

CATGCGAAGGAGGCAAGGGATGCAATCAACACAGTCTTGGGCGACTTTTATAAGATCGAT
 H  A  K  E  A  R  D  A  I  N  T  V  L  G  D  F  Y  K  I  D

AGGACTCCAATTCTGAAAGCAATGTGGAGAGAGGCCAAGGAATGCATCTTCATCGAGCCT
 R  T  P  I  L  K  A  M  W  R  E  A  K  E  C  I  F  I  E  P

GAAAAAGGTAGGGAGTCCAAGGGTGTATATTGGTACAATAAATTCTGA
 E  K  G  R  E  S  K  G  V  Y  W  Y  N  K  F  *
```

CLAIMS

1. A method of producing acetylenic compounds, characterized in that a double bond is converted to an acetylenic bond by an acetylenase.

2. A method according to claim 1, wherein acetylenic fatty acids are produced by conversion of unsaturated fatty acids to acetylenic fatty acids by a fatty acid acetylenase.

3. A method according to claim 2, wherein C18 fatty acids with doublebonds at position delta 12 are converted to 12-ynoic acids.

4. A method according to claim 3, wherein linoleic acid is converted to crepenynic acid (9-octadecen-12-ynoic acid) by *Crepis alpina* delta 12 acetylenase.

5. cDNA coding for acetylenase of the mixed function monoxygenase type containing three conserved histidin motifs ($HX_{(3\text{ or }4)}H$, $HX_{(2\text{ or }3)}HH$, and $HX_{(2\text{ or }3)}HH$) according to Sequence Listing 1.

6. cDNA according to claim 5 encoding fatty acid acetylenase.

7. cDNA according to claim 6 encoding *Crepis alpina* delta 12 acetylenase comprising the sequence according to Sequence Listing 2 or any nucleotide sequences essentially homologous therewith.

8. Use of cDNA according to any of the claims 5, 6 or 7 for transforming organisms.

9. Use according to claim 8, wherein the organisms will be capable of accumulating acetylenic compound.

10. Use according to claim 8, wherein the organisms are oil accumulating organisms.

11. Use according to claims 10, wherein the oil accumulating organisms are selected from the group consisting of oil crops, oleogeneous yeasts and moulds.

12. Organisms transformed with a acetylenase cDNA according to any of the claims 5, 6 or 7.

13. Organisms according to claim 12, which are organisms accumulating acetylenic compounds.

14. Organisms according to claim 12, which are organisms accumulating oil.

15. Organisms according to claim 14, which are selected from the group consisting of oil crops, oleogeneous yeasts and moulds.

16. Acetylenic componds accumulated in organisms according to claim 13.

17. Oils from oil accumulating organisms according to claims 14 or 15.

AMENDED CLAIMS

[received by the International Bureau on 30 July 1997 (30.07.97); original claims 1-17 replaced by amended claims 1-18 (2 pages)]

1. A method of producing acetylenic compounds, characterized in that C18 fatty acids with doublebonds at position delta 12 are converted to 12-ynoic acids by an acetylenase.

2. A method according to claim 1, wherein linoleic acid is converted to crepenynic acid (9-octadecen-12-ynoic acid) by *Crepis alpina* delta 12 acetylenase.

3. A DNA sequence coding for acetylenase of the mixed function monoxygenase type containing three conserved histidin motifs ($HX_{(3\ or\ 4)}H$, $HX_{(2\ or\ 3)}HH$, and $HX_{(2\ or\ 3)}HH$) according to Sequence Listing 1.

4. A DNA sequence according to claim 3 encoding fatty acid acetylenase.

5. A DNA sequence according to claim 4 encoding a delta 12 fatty acid acetylenase.

6. A DNA sequence according to claim 5 encoding *Crepis alpina* delta 12 acetylenase comprising the sequence according to Sequence Listing 2 or any nucleotide sequences encoding an acetylenase essentially homologous therewith.

7. Use of a DNA sequence according to any of the claims 3, 4, 5 or 6 for transforming organisms.

8. Use according to claim 7, wherein the organisms will be capable of accumulating acetylenic compound.

9. Use according to claim 7, wherein the organisms are oil accumulating organisms.

10. Use according to claim 9, wherein the oil accumulating organisms are selected from the group consisting of oil crops, oleogeneous yeasts and moulds.

11. Organisms transformed with an acetylenase DNA according to any of the claims 3, 4, 5 or 6.

12. Organisms according to claim 11, which are organisms accumulating acetylenic compounds.

13. Organisms according to claim 11, which are organisms accumulating oil.

14. Organisms according to claim 13, which are selected from the group consisting of oil crops, oleogeneous yeasts and moulds.

15. A method of obtaining acetylenic componds, comprising accumulation of acetylenic compunds in organisms according to claim 12.

16. A method of obtaining oils, comprising accumulation of oils in organisms according to claims 13 or 14.

17. Acetylenic compounds obtainable by the method according to claim 15.

18. Oils obtainable by the method according to claim 16.

FIG. 1

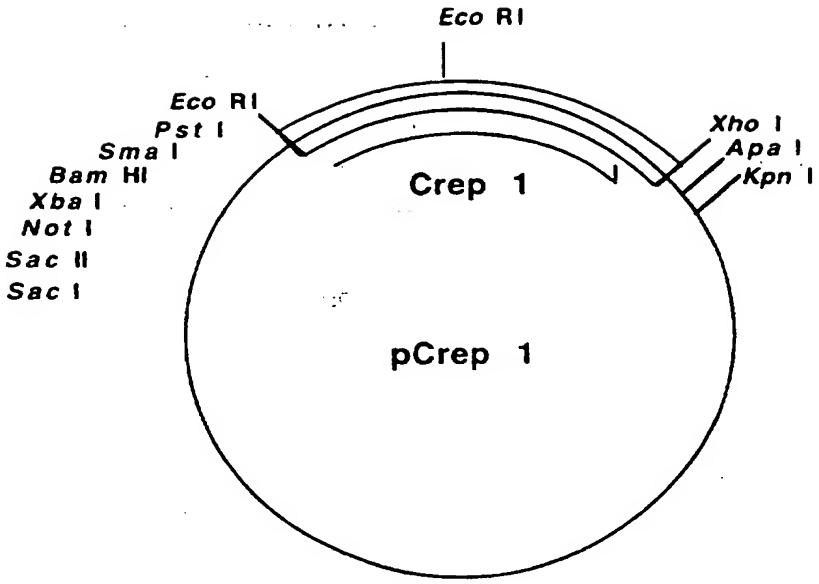


FIG. 2

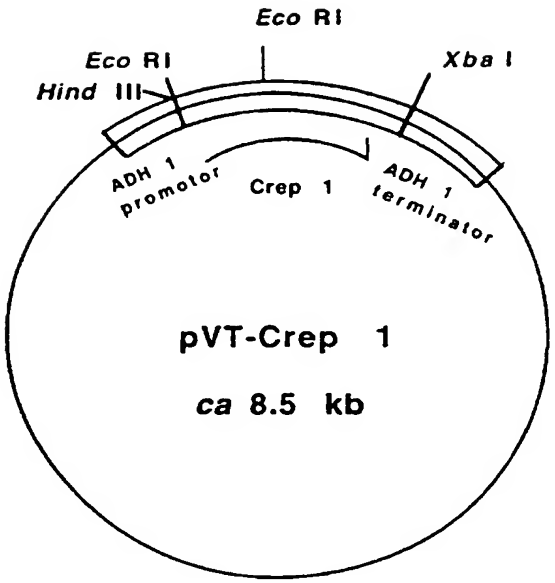


FIG. 3

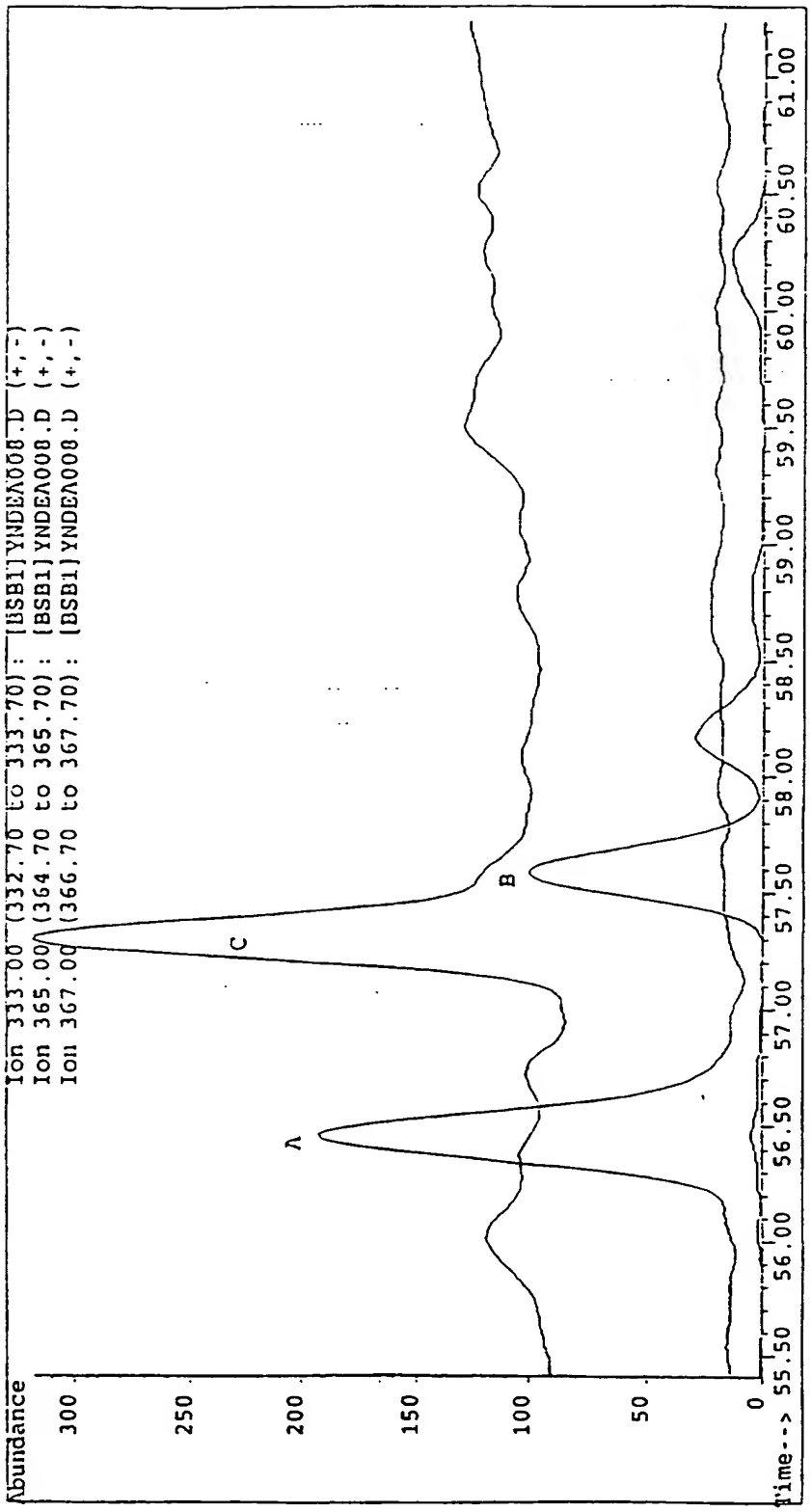


FIG. 4

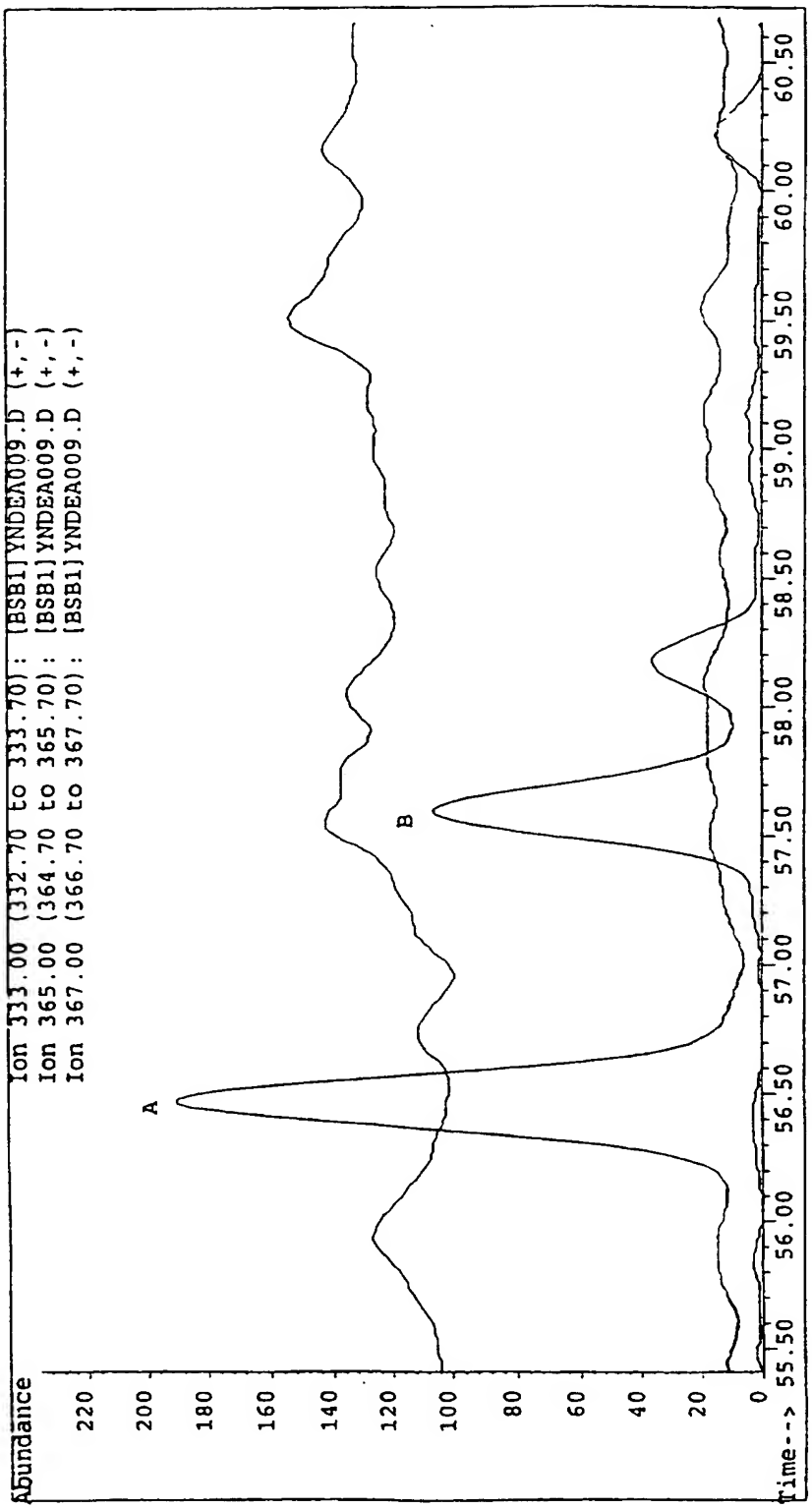


FIG. 5

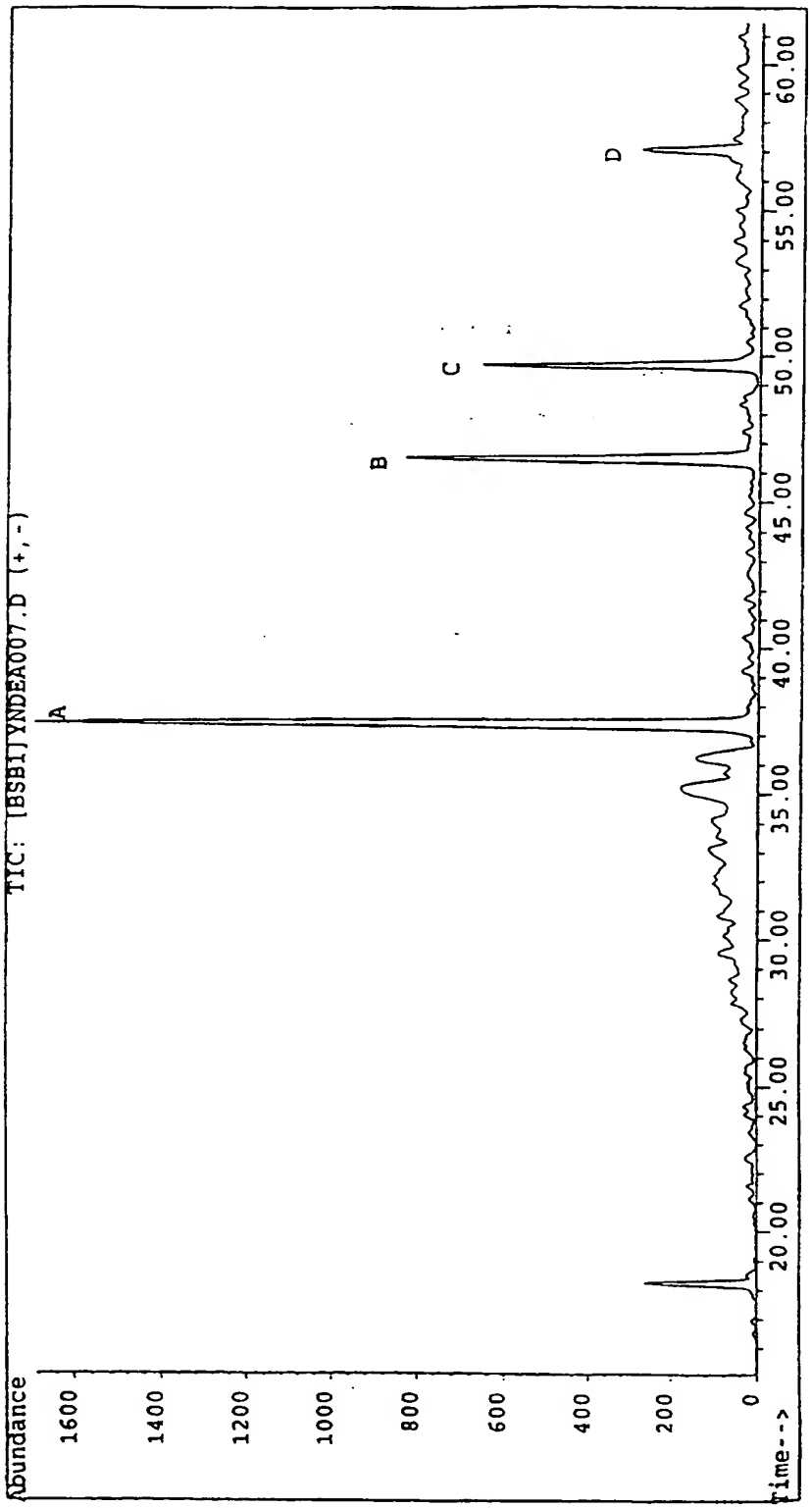
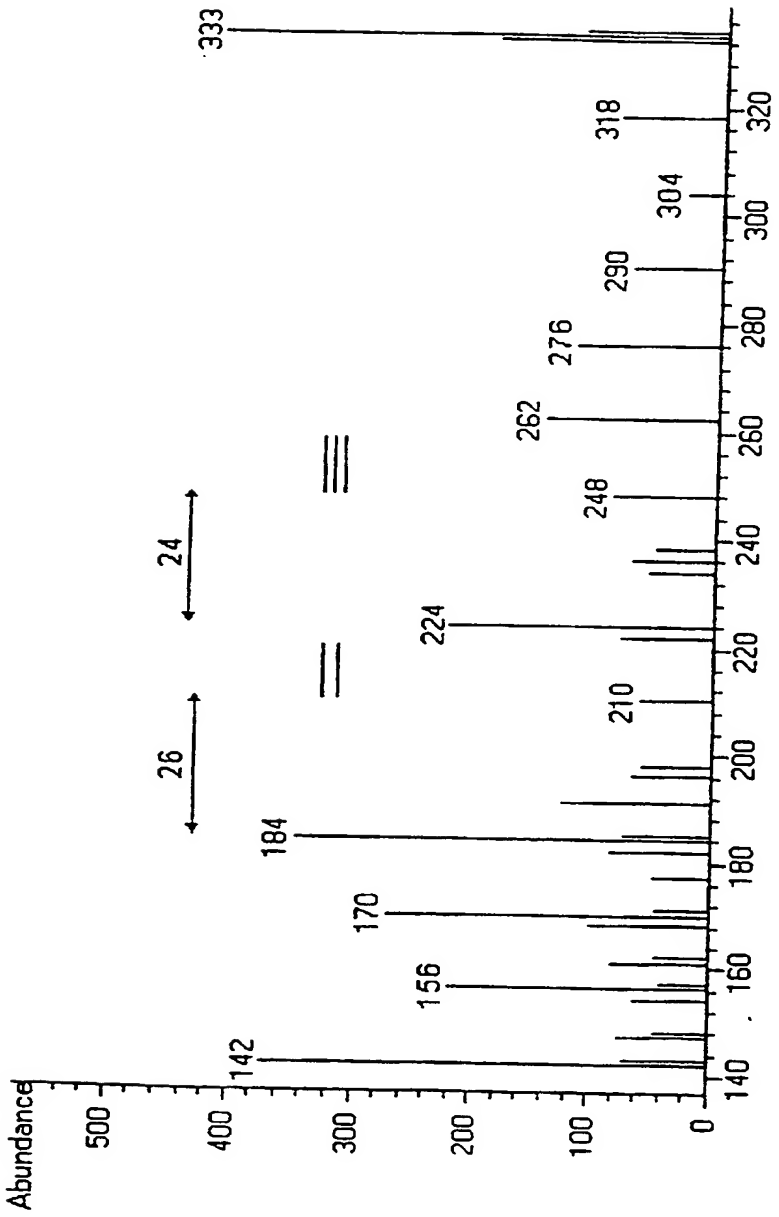


FIG. 6



INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 97/00247

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12P 7/64, A01H 5/10, C12N 9/02
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CAPLUS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J. Plant Physiol., Volume 144, 1994, Gerhard Kohn et al, "Biosynthesis of Acetylenic Fatty Acids in the Moss Ceratodon purpureus (Hedw.) Brid" page 265 - page 271 --	1-2
X	Lipids, Volume 3, No 4, 1968, W.G. Haigh et al, "Acetylenic Acid Biosynthesis in Crepis rubra" page 307 --	16-17
X	Biochim. Biophys. Acta, Volume 137, 1967, Bu'Lock, "The biosynthesis of an acetylenic acid, crepenynic acid" page 391 - page 392 -- --------	16-17

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
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"&" document member of the same patent family

Date of the actual completion of the internati nal search	Date of mailing of the international search report
20 May 1997	30-05-1997
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carolina Gómez Lagerlöf Telephone No. +46 8 782 25 00

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